Chapter 11

Viral Engineering of Chimeric Antigen Receptor Expression on Murine and Human T Lymphocytes

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Abstract

The adoptive transfer of a bolus of tumor-specific T lymphocytes into cancer patients is a promising therapeutic strategy. In one approach, tumor specificity is conferred upon T cells via engineering expression of exogenous receptors, such as chimeric antigen receptors (CARs). Here, we describe the generation and production of both murine and human CAR-engineered T lymphocytes using retroviruses.

Key words Chimeric antigen receptor, T lymphocytes, CAR-T cell, Gamma-retrovirus, Lentivirus

1 Introduction

Cancer immunotherapy aims to utilize or bolster the immune system's antitumor capabilities to regress or cure malignancies. One such strategy sees cancer patients treated with a bolus of their own T cells possessing specificity against their tumor. These adoptive T cell therapies have seen significant clinical success in a variety of malignancies [1–5]. Obtaining tumor-specific T cells on a per-patient basis is afforded through either the expansion of endogenously occurring tumor-specific T cells or by genetically engineering peripheral T cells to express receptors with specificity for tumor targets. Both of these strategies require the generation of large numbers of tumor-specific T cells prior to treatment through an ex vivo culture period. Chimeric antigen receptors (CARs) are recombinant proteins composed of an extracellular antigen recognition domain and an intracellular activation domain, which trigger T cell activation and cytotoxicity upon binding of target antigen [6]. Here, we focus on the production of both murine and human T cells engineered to express a CAR for preclinical evaluation.

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Josie Ursini-Siegel and Nicole Beauchemin (eds.), *The Tumor Microenvironment: Methods and Protocols*, Methods in Molecular Biology, vol. 1458, DOI 10.1007/978-1-4939-3801-8_11,

Genetic engineering of recombinant receptors in T lymphocytes is most commonly accomplished using retroviruses. Retroviruses, members of the *Retroviridae* family of enveloped viruses, have the natural capacity to integrate their genetic information into the genome of infected host cells. As such, a retrovirus engineered to encode a CAR can permit the production of T cells which stably express the tumor-specific receptor. *The protocol described herein details the production of two different retroviruses and their use for the production of CAR-T cells: a gamma-retrovirus with tropism for murine cells (used for the generation of murine CAR-T cells) and a lentivirus with tropism for human cells (used for the generation of human CAR-T cells).*

Production of a gamma-retrovirus [7] is accomplished by combining a retroviral vector (encoding the CAR in a retroviral genomic backbone) with a packaging plasmid and/or packaging cell line (both of which contain the *gag*, *pol*, and *env* genes required for production of functional viral particles). By providing the viral packaging genes *gag*, *pol*, and *env* in *trans* (not encoded in the viral genome), the resultant gamma-retroviruses are non-replicative, increasing the safety profile of the system. Here, we co-transfect the Platinum-E (Plat-E) packaging cell line [8] with our retroviral vector and the packaging plasmid pCL-Eco [9] to generate gamma-retroviruses.

Similar to the gammaretrovirus used for transduction of murine cells, the lentivirus utilized for transduction of human T cells is prepared using a conditional, split-genome packaging system that is based off of the HIV-1 backbone [10]. This platform dispenses with all virulent and non-essential genes, and uses complementation of *gag, pol, rev*, and the vesicular stomatitis virus envelope protein in *trans* to generate a self-inactivating, non-replicative virion. In this protocol, we utilize the pRSV-REV and pCCL packaging and transfer vectors, respectively.

2 Materials

2.1 Tissue Culture Considerations

All methods should be conducted in a BSL2 certified biological safety cabinet to ensure sterility and safety. Use filter tips anytime you are pipetting retroviruses (gamma-retrovirus or lentivirus) or retrovirally transduced cells to prevent contamination of pipettes with viral particles. Tissue culture incubation conditions are 37 °C, 5% CO_2 and ambient O_2. Reagents for tissue culture and virus work should all be stored at 4 °C and preheated in a 37 °C water bath prior to use.

2.2 Common Materials and Reagents

1. BSL2-certified biological safety cabinet.
2. Tabletop centrifuge.
3. 1× phosphate buffered saline (PBS).

4. T cell media: RPMI 1640, 10% heat-inactivated fetal bovine serum (hiFBS), 2 mM L-glutamine, 10 mM HEPES, 0.5 mM sodium pyruvate, 1× nonessential amino acids, 55 μM β-mercaptoethanol, 100 U/mL penicillin + 100 μg/mL streptomycin.

2.3 Gamma-Retrovirus Preparation

1. T-75 tissue culture coated flasks.
2. Platinum-E (Plat-E) cells.
3. Plat-E maintenance media: Dulbecco's Modified Eagle Medium (DMEM), 10% hiFBS, 2 mM L-glutamine, 10 mM HEPES, 10 μg/mL blasticidin, 1 μg/mL puromycin, 0.1 mg/mL normocin.
4. Plat-E transfection media: DMEM, 10% hiFBS, 2 mM L-glutamine, 10 mM HEPES, 0.1 mg/mL normocin.
5. 15 mL Falcon tubes.
6. Opti-MEM media.
7. Retroviral plasmids; packaging (pCL-Eco [9]) and transfer plasmid (e.g., pRV2011 and pRV100G, however, other retroviral transfer vectors may be substituted) engineered to contain your gene of interest (CAR).
8. Lipofectamine 2000 (Life Technologies) (*see* **Note 1**).
9. 15 mL 100K NMWL centrifugal filter.
10. 10 mL syringe.
11. 0.45 μm syringe filter.

2.4 Murine CAR-T Cell Preparation

1. Murine donor.
2. Tweezers.
3. Small, sharp scissors.
4. 70% ethanol.
5. 6 cm petri dishes.
6. Microscope slides, with frosted glass tips.
7. Transfer pipettes.
8. 24-well, tissue culture coated plates.
9. ACK lysis buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (prepared with autoclaved Milli-Q water).
10. Recombinant human IL-2.
11. Anti-murine CD3e, clone: 145-2C11.
12. Anti-murine CD28, clone: 37.51.
13. Polybrene.

2.5 Lentivirus Preparation

1. HEK293T cells.
2. HEK293T maintenance media: DMEM, 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin + 100 μg/mL streptomycin.

3. HEK293T transfection media: DMEM, 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/mL normocin (*see* **Note 1**).
4. T-150 tissue culture coated flasks.
5. 15 cm dish.
6. 1× trypsin–EDTA solution (0.05%), no phenol red.
7. Lentiviral transfection plasmids: pRSV-Rev, pMDLg-pRRE, pMD2.G, pCCL/pRRL transfer plasmid.
8. Lipofectamine 2000.
9. Opti-MEM media.
10. 1 M sodium butyrate.
11. 150 mL 0.45 μm cellulose acetate or polyenersulfone (PES) Filter.
12. Ultracentrifuge with appropriate rotor and tubes suitable for ~130k RCF and ~40 mL (such as the Beckman Coulter SW-32 Ti Swinging Bucket Rotor and 38.5 mL Ultracentrifuge tubes).
13. 24-well, tissue culture coated plates.

2.6 Human CAR-T Cell Generation

1. PBMCs isolated from healthy donors.
2. Recombinant hIL-2.
3. Recombinant hIL-7.
4. Gibco Dynabeads Human T cell Activator CD3/CD28 beads and necessary magnets (e.g., Gibco Dynabeads and Dynabeads® MPC®-S) (Magnetic Particle Concentrator).
5. 1× PBS + 0.1% BSA + 2 mM EDTA.
6. 96-well round bottom, tissue culture coated plates.
7. 24-well, tissue culture coated plates.
8. T-25 and T-75 tissue culture coated flasks.

3 Methods

3.1 Murine CAR-T cells

3.1.1 Gamma-Retrovirus Generation

Note: Protocols 3.1.1 and 3.1.2 are interconnected in that the retrovirus generated in Subheading 3.1.1 will be used to transduce the murine T cells in Subheading 3.1.2; as such, the timelines used are consistent between the two protocols. Refer to Fig. 1 for a timeline of gamma-retrovirus production.

Preparation:

1. Seed a T-75 flask with retrovirus packaging Plat-E cells (*see* **Note 2**) in transfection media (*see* **Note 3**) such that the cells will be ~70% confluent at the time of transfection (*see* **Note 4**).

Day 1—transfect Plat-E cells:

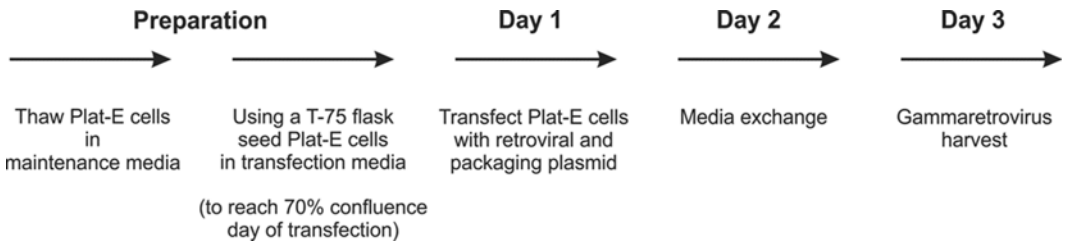

Fig. 1 Timeline of gammaretrovirus preparation. A visual representation providing a broad overview of the steps required/timeline for preparation of a CAR-encoding gammaretrovirus. The timeline of preparation steps may vary depending on density of Plat-E cell seeding and is thus unspecified

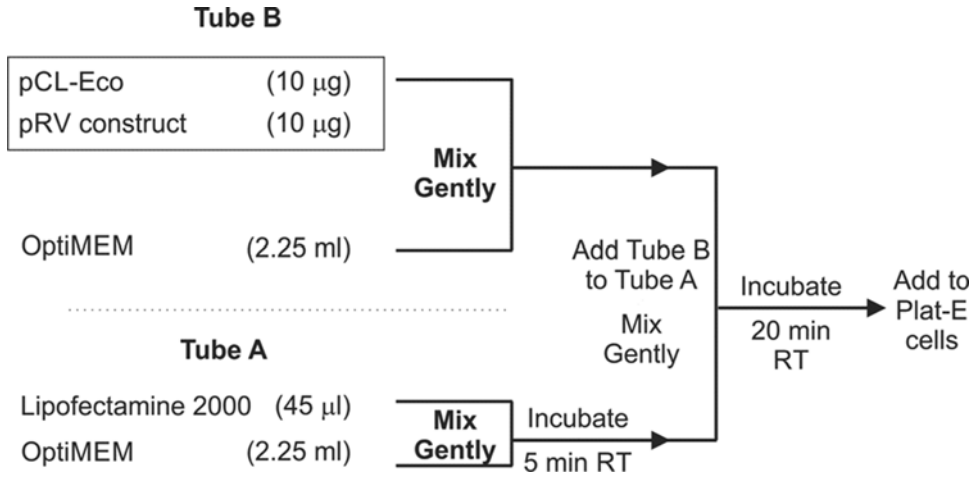

Fig. 2 Plat-E cell transfection. Flowchart indicating amounts and procedures for transfection of Plat-E cells for the generation of gammaretrovirus. To be completed on day 1 of the gammaretrovirus/murine CAR-T cell production process

2. Transfection should be conducted in the afternoon (*see* Fig. 2 for a visual summary). Prepare two 15 mL Falcon tubes each containing 2.25 mL of room temperature Opti-MEM media. To the first, Tube A, add 10 µg of retroviral plasmid DNA and 10 µg of packaging plasmid (pCL-Eco) DNA. To the second, Tube B, add 45 µL Lipofectamine 2000, vortex gently to mix, and allow to rest at room temperature for 5 min (*see* **Note 5**). Add Tube B to Tube A, vortex gently to mix, and incubate at room temperature for 20 min. Add the 4.5 mL of DNA/Lipofectamine Opti-MEM solution to the T-75 Plat-E flask; mix by gently rocking the flask front-to-back and side-to-side (*see* **Note 6**). Incubate at 37 °C and 5% CO_2 overnight.

Day 2—change media:

3. The next morning, carefully aspirate off the 14.5 mL of media from the transfected Plat-E flask. Replace with 10 mL of fresh,

pre-warmed transfection media being cautious not to disturb the cell layer.

Day 3—harvest viral supernatants:

4. The next morning, wash an Amicon Ultra 100K centrifugal filter; add 5 mL of PBS to the upper chamber and centrifuge at 2000 × *g* for 5 min. Aspirate flow through from the lower chamber. You will require one filter per Plat-E flask.
5. Collect supernatant from T-75 Plat-E flask. Load into a 10 mL syringe. Pass through a 0.45 μm syringe filter and into the upper chamber of a washed centrifugal filter. Concentrate viral supernatants 10× by centrifuging filters at 2000 × *g* for ~7–9 min; concentrate 10 mL of supernatant down to 1 mL.

3.1.2 Murine CAR-T Cell Generation

Refer to Fig. 3 for a visual summary of murine CAR-T cell production steps.

Day 2—activate splenocytes:

1. Harvest donor spleen as a source of murine T cells (*see* **Note** 7), working in a biological safety cabinet to ensure sterility. Saturate all instrumentation and the fur on the left side of the mouse with 70% ethanol. Using tweezers, lift the skin approximately midway between the front and hind limbs and make a long incision with sharp scissors. Locate the spleen beneath the peritoneum (a deep red organ with an elongated kidney shape). Cut into the peritoneal cavity. Use tweezers to carefully lift the spleen whilst using scissors to liberate the organ, cutting away any fatty material. Once removed, place the spleen in ~8 mL of T cell media in a 15 mL Falcon tube and keep on ice.
2. Decant Falcon tube contents (spleen and media) into a 6 cm petri dish. Using two scored microscope slides (use one microscope slide to score the frosted area of the other slide in a rough crosshatch pattern and vice versa), sandwich the spleen between the scored areas, using pressure and swirling one slide against the other to compress the spleen. Complete this step above the Petri dish, rinsing the slides with the media in the petri dish as necessary, to collect splenocytes. Continue rinsing and squishing until all that remains of the spleen is a small amount of white pulp, which can be discarded.
3. Eliminate large debris. Use a transfer pipette to collect the ~8 mL of media containing the liberated splenocytes into a 15 mL Falcon tube. Allow contents to settle for ~1 min. Transfer the supernatant (to avoid any sediment leave ~0.5 mL of media behind) into a fresh 15 mL Falcon tube.
4. Pellet cells (splenocytes) via centrifugation; 500 × *g*, 5 min. Aspirate the supernatant, being careful not to disturb the cell pellet.

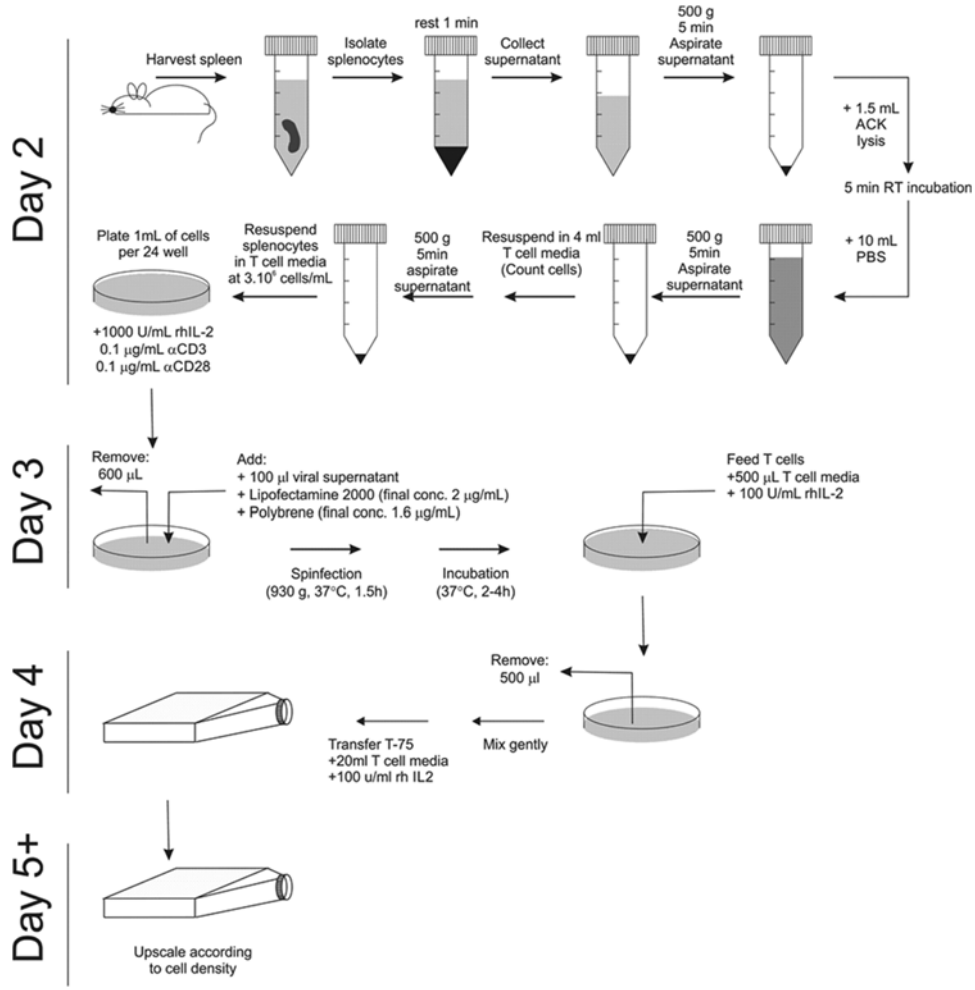

Fig. 3 Murine CAR-T cell generation. A flowchart which visually summarizes the steps/timeline required for the generation of CAR-engineered murine T cells; details are provided in the text

5. To lyse red blood cells, resuspend the pellet in 1.5 mL of ACK buffer. Incubate at room temperature for 5 min. Add 10 mL of PBS and pellet (500 × *g*, 5 min). Aspirate the supernatant.
6. Resuspend cell pellet in 4 mL of T cell media. Count cells.
7. Rinse: pellet cells (500 × *g*, 5 min), aspirate supernatant, and resuspend in T cell media as desired.
8. Plate splenocytes in a 24-well tissue culture dish; 3×10^6 splenocytes per well in 1 mL T cell media containing 100 IU/mL recombinant human IL-2, 0.1 µg/mL anti-CD3, and 0.1 µg/mL anti-CD28 (*see* **Notes 8** and **9**).
9. Incubate for 22–24 h at 37 °C and 5% CO_2.

Day 3—transduce splenocytes:

10. Remove 600 µL of media per well, being careful not to disturb the cell pellet.
11. Per well, add 100 µL of viral supernatant concentrate (from Subheading 3.1.1), Lipofectamine 2000 to 2 µg/mL, and Polybrene to 1.6 µg/mL.
12. Spinfection: centrifuge the 24-well plate at 930 × *g*, 37 °C, for 1.5 h.
13. Remove plate from centrifuge and incubate at 37 °C, 5% CO_2 for 2–4 h.
14. Add 500 µL of T cell media (return to 1 mL total volume per well) supplemented with recombinant human IL-2 at 100 IU/mL (use the same concentration as in **step 8**).

Day 4—expand T cell cultures:

15. Remove 500 µL of media from each well. Transfer remaining 500 µL (*see* **Note 10**) (pipette up and down gently to mix) into 20 mL of T cell media supplemented with recombinant human IL-2 (100 IU/mL, as in **steps 8** and **14**) in a T-75 flask (*see* **Note 11**).
16. Lay flask flat and incubate at 37 °C and 5% CO_2.

Ongoing—feed T cells:

17. As necessary, add T cell media supplemented with recombinant human IL-2 (100 IU/mL, as in **steps 8**, **14**, and **15**), keeping T cells at a concentration of 1–1.5 × 10^6 cells/mL (*see* **Notes 12** and **13**).
18. It is recommended to verify successful CAR-T cells generation by appropriate analytical methods, such as flow cytometric analysis of reporter genes encoded within the retrovirus (*see* **Notes 14–16**).

3.2 Human CAR-T Cells

3.2.1 Lentivirus Preparation

Refer to Fig. 4 for timeline of lentivirus production. Ideally, the transfer plasmid used to encode your CAR will also contain some form of transduction marker, such as a fluorescent protein (e.g., GFP) or a truncated receptor (e.g., nerve growth factor receptor), as this is needed for titrating the number of transducing units of lentivirus for the transduction of T cells.

Day –3—Thaw HEK293T cells for seeding:

1. Thaw cryopreserved HEK293T cells in 37 °C water bath (*see* **Note 18**) and seed roughly 1.5–2 × 10^6 cells in 20 mL HEK293T maintenance media per T-150 flask. On average, two confluent T-150 flasks are required to obtain enough cells for one viral batch.

Day 0—Harvest HEK293T cells for plating:

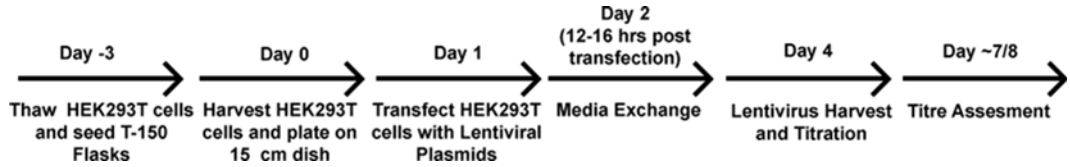

Fig. 4 Timeline of lentivirus preparation. A visual representation providing a broad overview of the steps required/timeline for preparation of a CAR-encoding lentivirus

2. Once HEK293T cells are ~90% confluent in the flask (after ~3 days), harvest HEK293T cells from flasks by first carefully aspirating media. Then flush residual media by gently washing cells with 5 mL pre-warmed 1× PBS (*see* **Note 19**).
3. Add 3 mL pre-warmed 1× trypsin–EDTA (0.05%) per T-150 flask and ensure coverage of cells. Incubate in a 37 °C incubator for 5 min (*see* **Note 20**). Add 7 mL HEK293T maintenance media to inactivate the trypsin–EDTA and combine cells from flasks.
4. Count cells by hemocytometer and resuspend HEK293T cells at 8×10^6 cells/mL in HEK293T maintenance media. Plate 1 mL of cells (i.e., 8×10^6) + 19 mL HEK293T culture media per 15 cm dish, for a total of three 15 cm dishes. Seed one T-150 flask with 0.5×10^6 cells to be used for titration purposes later on. Place flask and dishes into 37 °C incubator overnight (*see* **Note 21**).

Day 1—Transfect HEK293T cells:

5. One hour prior to transfection (*see* **Note 22**), replace the media on each plate carefully (*see* **Note 23**) with 12 mL of HEK293T transfection media containing an antibiotic suitable for your transfection reagent.
6. Transfection with Lipofectamine 2000 (*see* Fig. 5 for a visual summary) utilizes 60 μg DNA with 2 μL Lipofectamine per μg DNA. Prepare two series of 15 mL screw cap tubes for each plate as outlined in Fig. 2 (*see* **Note 24**). Once the Lipofectamine with DNA is added, gently swirly the dish to evenly distribute transfection reagents. Place the dish into the 37 °C incubator (*see* **Note 25**).

Day 2—Media Change:

7. Following transfection, roughly 12–16 h later, change the media on each dish carefully (see Note 23) with 12 mL HEK293T maintenance media supplemented with sodium butyrate (1 mM final). Place the dishes back into the 37 °C incubator to incubate for 48 h.

Day 4—Lentivirus Harvest:

8. Prior to viral harvest, precool ultracentrifuge buckets and rotor to 4 °C (*see* **Note 26**).
9. Harvest the media from the three 15 cm dishes into a 50 mL screw cap tube and centrifuge at 2000 RCF_{max} for 10 min to sediment any collected HEK293T cellular debris.
10. Filter the supernatant through a 0.45 μm PES filter (*see* **Note 27**) and add the filtrate to ultracentrifuge tubes, toping up with enough cold 1× PBS to bring within 0.5 cm of the tube (*see* **Note 28**).
11. Create any necessary balance tubes and pellet the lentivirus at RCF_{max} of 130,000 for 1 h 40 min, 4 °C, with minimal deceleration (*see* **Note 29**).
12. During the ultracentrifugation, harvest HEK293T cells from the T-150 flask seeded on day 1 as previously laid out. Plate 30,000 cells in 0.5 mL HEK293T maintenance medium (6×10^4 cells/mL) per well of a 24-well plate (*see* **Note 30**) for the purposes of lentivirus titration, and place in a 37 °C incubator to adhere for 3 h.
13. Once centrifuged, dispense supernatant into a bleach bucket. Keeping the ultracentrifuge tube inverted, place onto several layers of paper towel for 2 min to remove residual supernatant (*see* **Note 31**).
14. Add 60 μL ice-cold 1× PBS to the ultracentrifuge tube and place on ice for 10 min (*see* **Note 32**).
15. Begin resuspension by slowly and gently scraping the bottom of the ultracentrifuge tube using a micropipettor (*see* **Note 33**). Continue doing so until the pellet is mostly taken off, then begin slowly pipetting up and down gently to resuspend (*see* **Note 34**).

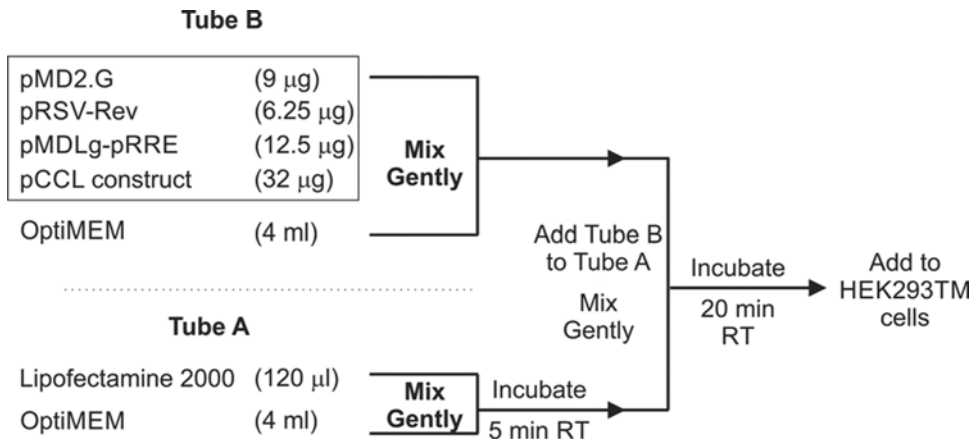

Fig. 5 HEK293T cell transfection. Flowchart indicating amounts and procedures for transfection of HEK293T cells for the generation of lentivirus. To be completed on day 1 of the lentivirus/human CAR-T cell production process

16. Once the pellet has begun to dissolve add more ice-cold 1× PBS (*see* **Note 19**) and transfer to a 1.5 mL microcentrifuge tube and resuspend until adequately homogenous (*see* **Note 35**).
17. Make aliquots into 1.5 mL microcentrifuge tubes, making sure to make a separate aliquot of 3 μL for the purposes of titration (*see* **Note 36**). Place aliquots into −80 °C and proceed to decontaminate centrifuge buckets and anything that has come into contact with virus with 70% ethanol.
18. Once HEK293T cells have adhered for at least 3 h, prepare a series of dilutions using the virus aliquot frozen for titration (refer to Table 1 and *see* **Note 38**).
19. Add 500 μL of dilution to a corresponding well of the 24-well plate. Two wells will receive only HEK293T maintenance media to act as either the unstained control, or stained non-transduced control. Allows cells to grow for 2–3 days.

Day 7/8—Titer Determination:

20. Harvest HEK293T cells from wells into 5 mL polystyrene tubes using a micropipettor with filter tips.
21. Stain cells using a fluorochrome-conjugated antibody specific for the transduction marker included in the transfer plasmid, or if the vector encodes a fluorescent molecule such as GFP simply run cells on the flow cytometer (*see* **Notes 39** and **40**).
22. Calculate the titer based on the dilution resulting in ~10% positive cells. Your flow data should be gated on the marker$^+$ population by histogram on your stained non-transduced cells. Then subtract the %marker$^+$ of the stained non-transduced from the rest of your samples.

    (a) Calculation: $30{,}000 \times \text{positive fraction} \times \text{dilution factor} = \text{virus titer in TU/mL}$.

    For example, for 17% marker$^+$ in 10^{-5} dilution.

    $30{,}000 \times 0.17 \times 100{,}000 = 5.1 \times 10^8$ TU/mL.

3.2.2 Human CAR-T Cell Generation

In general, T cell culture media is to be supplemented with 100 U/mL IL-2 and 10 ng/mL IL-7 immediately before use with primary T cells (*see* **Note 40**). Refer to Fig. 6 for a visual summary of human CAR-T cell production steps.

1. Human PBMCs isolated from healthy individuals should be acquired before beginning this protocol. These cells can be bought from commercial vendors (e.g., Normal LeukoPack; HemaCare Corporation) or derived from donors on site using various established methods (e.g., Ficoll-Paque Plus gradient separation [11]). Such protocols are readily available online through the manufacturer website.

Table 1
Example dilutions for lentiviral titration

Final dilution (after addition to cells)	Volume of virus (μL)	Diluent to add (μL)
1×10^{-3}	2 of stock	998
1×10^{-4}	70 of previous dilution	630
1×10^{-5}	70 of previous dilution	630
1×10^{-6}	70 of previous dilution	630

Frozen and thawed lentiviral stocks are diluted in HEK293T maintenance media as below; 500 μL of each dilution is added to HEK293T cells plated in 500 μL in 24-well plates, resulting in final dilutions. Other dilutions may be utilized to provide greater accuracy as per your individual virus

2. Thaw human PBMCs in 37 °C water bath, then slowly add cells drop wise to 7 mL T cell media (with no cytokine supplementation) (*see* **Note 41**). Mix gently and remove 10 μL to count cells by trypan exclusion on a hemocytometer. In parallel, spin the remaining cells at 500 × *g* for 5 min.
3. Carefully aspirate media by tilting tube horizontal so as not to disturb pellet. Resuspend cells to a final concentration of 1×10^6 cells/mL.
4. If you desire to use isolated total T cells, or CD8 or CD4 only T cells, use an appropriate means of sorting such as FACS or column purification. We suggest negative selection sorting columns.
5. To begin cultures start with 100,000 PBMCs per well of a round bottom 96-well plate. Take 100 μL of the PBMCs prepared in **step 3**, with one additional well for security (*see* **Notes 42**).
6. anti-CD3/anti-CD28-coated Dynabeads are utilized to activate T cells within the PBMC mixture and begin their proliferation (*see* **Note 43**). Following the manufacturer guidelines, briefly vortex the beads for 30 s and transfer 2 μL beads per well to a 1.5 mL microcentrifuge tube (*see* **Note 44**). Add 1 mL PBS + 0.1 % BSA + 2 mM EDTA to beads, vortex for 5 s.
7. Place beads on MPC-S magnet for 1 min, then aspirate the supernatant with care taken to not disrupt the beads gathered on the side of the 1.5 mL tube.
8. Remove 1.5 mL tube from the magnet and resuspend the beads with 100 μL T cell media supplemented with 2× cytokine (200 U/mL IL-2 and 20 ng/mL IL-7), referred to as activation media (2×), per 2 μL beads.
9. Aliquot 100 μL PBMC (100,000 cells) per well of a round-bottom 96-well dish. Combine with 100 μL of Dynabead activator solution previously made, per well. This results in the

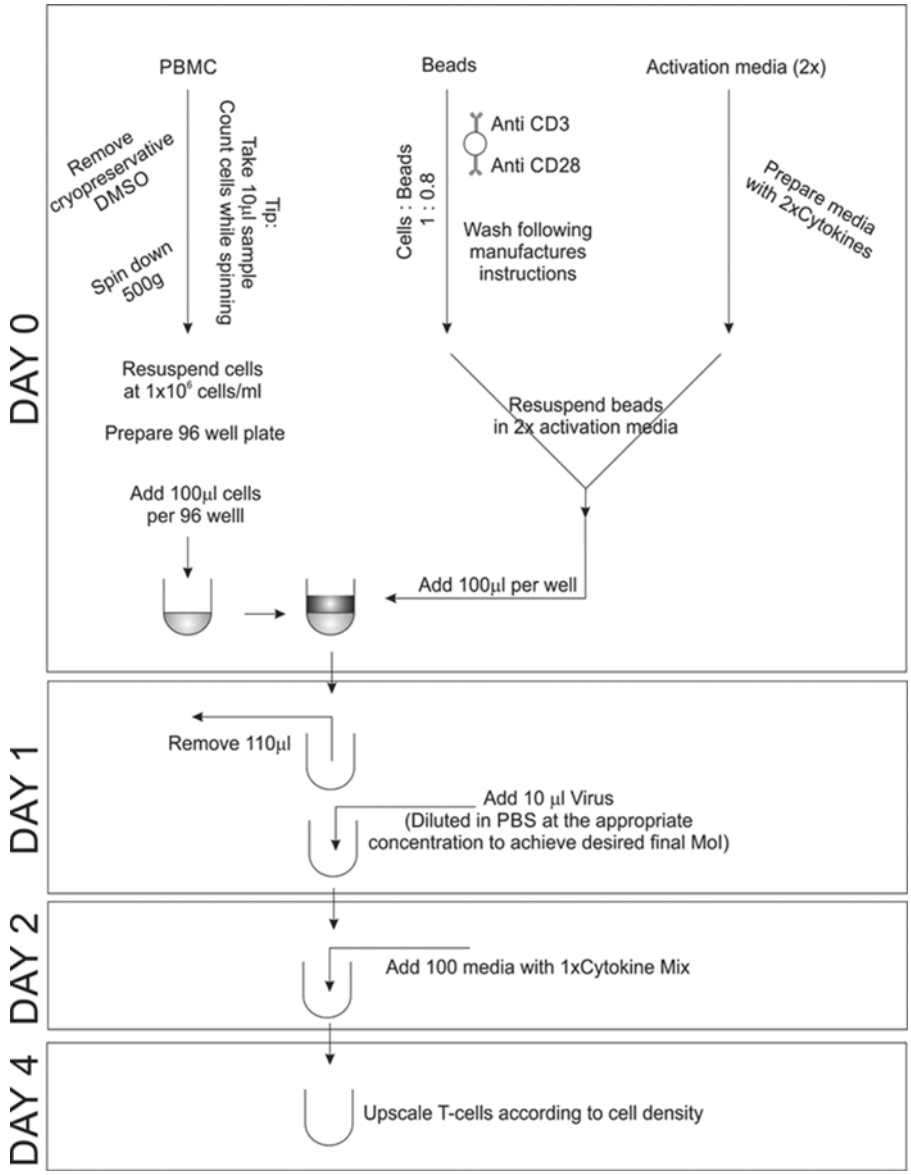

Fig. 6 Human CAR-T cell generation. A flowchart which visually summarizes the steps/timeline required for the generation of CAR-engineered human T cells, details of which are provided in the text

final cytokine concentration of 100 U/mL IL-2 and 10 ng/mL IL-7. Place into 37 °C incubator overnight.

10. After 18–24 h of incubation use the titer of the virus previously created and transduce the culture at the desired MOI (*see* **Note 45**). To do so, remove 110 µL from each well carefully (*see* **Note 46**). Take necessary amount of virus for desired MOI, bring up to 10 µL with PBS if below 10 µL, and add to cells gently without disturbing the pellet. Place into 37 °C incubator overnight.

11. The following day, supplement the wells with 100 μL T cell media supplemented with IL-2 and IL-7.
12. Check cells every day to assess growth. T cells will appear as a white halo surrounding a small red core of beads. When the halo reaches ~2–2.5 mm in diameter, the cells are ready to scale up (*see* **Note 47**).
13. Add 900 μL cytokine supplemented T cell medium per well of a 24-well plate corresponding to the number of the wells used at the 96-well stage. Transfer the ~200 μL cells into 900 μL cytokine supplemented (*see* **Note 48**).
14. Continue to culture the T cells in the 24-well plate until the wells are ~90–95% confluent (*see* **Note 49**). At this point, combine two wells and transfer to a T25 flask laying down, bringing up to 5 mL with fresh cytokine supplemented T cell media (*see* **Note 50**).
15. From this point on, cells should be given fresh media with cytokine every 2–3 days. Attention should be paid to the color of the media, as well as cell density. Thus, cells should be counted every 2–3 days and fed accordingly to maintain a density of ~1.0×10^6 cells/mL. If cells are growing well, the media will begin to turn yellow and will need to be supplemented with fresh media.
16. Cells can be scaled to larger flasks as they increase in number. While maintaining roughly ~$0.1–2 \times 10^6$ cells/cm^2 and 1.0×10^6 cells/mL, when the cells reach sufficient number increase the flask size (*see* **Note 51**).

4 Notes

1. There are many commercial transfection reagents available. We recommend Lipofectamine as a high efficiency reagent. Others may be substituted in place, however the DNA to transfection reagent ratios must be optimized. When Lipofectamine 2000 reagent is utilized, penicillin/streptomycin interferes with the transfection processes. In place of penicillin/streptomycin, either normocin antibiotic or no antibiotic should be used during transfection. Other reagents, such as Lipofectamine 3000, may be less susceptible to this problem. However, we are not experienced with such reagents.
2. We keep Plat-E cells in culture for a maximum of 2 weeks before thawing a fresh batch. Decreased transduction efficiencies may be observed if gamma-retroviruses are generated from Plat-Es that have been in culture for extended periods.
3. When thawing and over extended periods in culture, Plat-E cells should be passaged in maintenance media. The puromycin

and blasticidin contained therein ensure Plat-E cells retain expression of the viral packaging genes *gag*, *pol*, and *env*. However, due to potential toxicities associated with increased cellular permeability to antibiotics during transfection, Plat-Es should be cultured in transfection media (containing normacin in lieu of puromycin and blasticidin) during this time.

4. In order to generate a T-75 flask of 70% confluent PLAT-E cells, a T-75 flask may be seeded with 1.15×10^6 Plat-E cells three d before, 2.3×10^6 Plat-E cells two d before, or 5.6×10^6 Plat-E cells the day before transfection. Alternatively, frozen vials of 2.8×10^6 Plat-E cells will generate a 70% confluent T-75 in 3 days post-thaw.
5. In our experience, efficiency of retrovirus generation is decreased if Opti-MEM DNA and Lipofectamine solutions are scaled up; prepare one set of two tubes (DNA and Lipofectamine solutions) per each T-75 flask of Plat-E cells being transfected.
6. One transfected Plat-E flask is sufficient to generate enough gamma-retrovirus for transduction of ten wells of T cells.
7. One spleen from a 6–8-week-old C57BL/6 or Balb/c mouse typically yields ~100 million splenocytes.
8. Gamma-retroviruses require actively dividing cells for efficient transduction; thus, the purified retrovirus is added to T cells 24 h post-activation with anti-CD3 and anti-CD28.
9. Altering the type and amount of cytokines in the T cell growth media can impact on the phenotype, and thus functionality, of T cells grown in vitro [12]; different cytokine compositions to yield a desired phenotype are available in the literature.
10. When observing the 24-well plate under a microscope, post-activation, you should expect to see dense clusters of T cell growth rather than a homogenous dispersion of cells throughout the well. In our experience these clusters are desirable for optimal T cell growth.
11. If preparing multiple wells of murine T cells simultaneously, three wells transduced with the same virus and grown under the same conditions can be expanded into 60 mL of appropriately supplemented T cell media in a T-150 flask laid flat.
12. In our experience, CAR-T cells are generally usable for in vitro assays/in vivo experiments from 4 to 7 days post-activation. After this point, viability of T cell cultures begins to rapidly decline.
13. Starting from 3×10^6 splenocytes, one well can yield upwards of 10^8 cells after expansion, depending on growth conditions. For example, we have observed yields of between 10 and 95×10^6 T cells 6 days after activation when growing splenocytes in 100 IU/mL rhIL-2. It should also be noted that in some cases, T cell growth rate may be dependent on the contents of your gamma-retrovirus.

14. We have observed that T cell cultures expanded from bulk splenocytes are between 80 and 95% pure for $CD4^+ + CD8^+$ cells; the distribution between $CD4^+$ and $CD8^+$ cells can range, for example, from 78.6% $CD8^+$ and 6.19% $CD4^+$ to 47.8% $CD8^+$ and 38.9% $CD4^+$ (generally $CD8^+$ T cells always dominate), depending on experimental variation and growth conditions.
15. It should be noted that it is possible to begin with a purified population of $CD4^+$ or $CD8^+$ T cells, rather than bulk PBMCs, if having an enriched final population of either is desirable for your purposes. We have found that magnetic negative selection kits work well for this purpose.
16. In our experience using this protocol, one can expect a transduction efficiency anywhere from 40 to upwards of 80%. This is generally dependent on the gamma-retrovirus used as well as experimental variability.
17. We have found that with our own HEK293T cells, usage of freshly thawed cultures of low passage, rather than older cultures, enhance lentiviral yield.
18. In our experience, HEK293T cells are weakly bound to the culture flask and so will lift off quite easily. Because of this, it is recommended that the media be removed carefully and the PBS wash to be done gently to prevent unnecessary loss of cells. To facilitate this, we suggest standing the flask up and aspirating the media away from the cells. Additionally, when adding PBS, add the volume to the flask upside down and then slowly rotate the flask so the PBS gently washes over the cells once. This is sufficient to remove residual media.
19. Although HEK293T cells can easily come off by mechanical means, the cells remain clumpy. Trypsin–EDTA is used to prevent clumping and facilitate accurate cell counting and even spreading on the 15 cm dish.
20. It is important that the dishes are level to ensure an even coverage of the dish with cells. We have found that three 15 cm dishes per viral batch are sufficient to yield high titer. However, this is optimized for the Beckman Coulter SW32Ti rotor and tubes capable of holding 38 mL. This may be adjusted per your needs and ultracentrifuge.
21. We suggest performing the transfection in the evening, such as 4 or 5 pm, to easily time the post-transfection media exchange, and lentivirus harvest.
22. It is important to be very gentle with exchanging media in the 15 cm dish. The HEK293T cells are easily lifted, thus we suggest removing and adding media using a pipette aid set to a slow setting if possible.
23. We have found that preparing a master mix of DNA with Lipofectamine reduces the efficiency of transfection. It is

therefore better to prepare a series of tubes for each dish. To increase productivity, you may make a master mix of the DNA to aliquot per tube.

24. At this point, the HEK293T cells will begin producing lentivirus upon transfection. It is important to follow all biosafety guidelines for BSL2 viruses that are in place in your institution.
25. It is essential that lentivirus be kept at 4 °C or on ice throughout the harvest once it has been pelleted, we have found this improves lentiviral titer.
26. A filter smaller than 0.45 μm, such as the commonly used 0.2 μm filter, has a pore size that is very close to the diameter of a lentivirus and will likely filter out virus. Similarly, cellulose acetate or PES membrane filters are recommended as nitrocellulose membranes will bind proteins on the lentivirus and bind up virions.
27. This is to ensure that the ultracentrifuge tube does not collapse during centrifugation.
28. Max deceleration may disrupt the lentivirus pellet and no brake requires a considerable period of time to fully stop that is not efficient.
29. Accurate counting is very important here as the math behind titration relies on the accuracy of plating 30,000 cells. Titration requires 6–7 wells per virus; however, it is beneficial to make spare wells in case of user error.
30. At this point, it is essential that the virus does not dry out and is kept at 4 °C to maintain a high titer of infective lentiviral particles. Thus, it is critical to ensure the tube is only kept inverted a minimal period of time and then promptly placed on ice.
31. From this point on, the virus should be on ice at all times to maintain stability during the resuspension process.
32. The ultracentrifugation process will have created a gel-like pellet. Scraping gently will assist in the resuspension by dislodging this gel. The micropipettor will naturally take up a small amount of liquid by doing this. It is important to be aware of this when resuspension by pipetting begins as it can introduce bubbles. The pellet will often resist complete resuspension and an emulsion is the best scenario at this point.
33. It is very important to avoid generating bubbles at any point during this step. We have found that exposure of lentivirus to bubbles will negatively impact yield. To prevent this from happening, we suggest setting the micropipettor to a lower volume.
34. The amount of 1× PBS added is up to the users' discretion and experience with assessing whether the yield is high titer or not. We find the best metric to follow is the turbidity of the resuspended solution. Increased turbidity suggests higher yield. Depending on your desired number of aliquots, volume per

aliquot, and titer, users can add from 40 to 100 μL more 1 × PBS. We suggest starting with 40 μL for the first batch.

35. Often there is a white "fluff" that is very difficult to fully resuspend, we have found that it does not impact the lentiviral efficacy if this is not fully homogeneous.
36. The volume of the aliquots is dependent on the titer of the virus. Thus, it is at your discretion and experience with the individual lentivirus being produced. We suggest 15 μL as a good middle ground.
37. When pipetting 2 μL, it is important to utilize an appropriate micropipettor to ensure high accuracy and low margin of error, such as a P2.
38. It should be noted that these cells have been exposed to lentivirus and as such should be handled accordingly under the necessary guidelines for your institution and state. Transduced cells are fixed with 4% glutaraldehyde to a final concentration of 2% for 20 min to inactivate lentivirus [13].
39. In addition to staining for the transduction marker, it may be beneficial to probe for expressed genes as well if compatible fluorochromes are available to determine the ability of the CAR to reach the cell surface.
40. The choice of cytokines is the determinant for much of the T-cell properties. It is a crucial choice that should be tailored to the scientists and experiments needs. For review, see DiGiusto and Cooper [14]. IL-2 and IL-7 aliquots should be frozen to −20 °C to maintain stability, and thawed just prior to use in fresh T cell medium.
41. Thawing cells rapidly at 37 °C enhances viability over a slower thaw. As the cryopreserved cells are stressed and have been maintained with DMSO, exposing cells to media slowly reduces osmotic shock.
42. We have found that starting with two wells (i.e., 200,000 cells) is sufficient to reach $15–100 \times 10^6$ cells by day 14 of culture, pending your specific construct. The limit of 100,000 cells is due to the limitations of lentivirus production. With higher titer virus, it may be possible to scale this step, however, we have not experimented with such a protocol. It should be noted that growth is highly construct-dependent, and is further affect by the MOI (higher results in slower growth).
43. VSV-G pseudotyped lentivirus is an excellent platform for transduction and gene insertion into non-dividing cells [15]. In regard to T cells, expression of the VSV-G receptor, LDL-receptor [16], is low until activation through the TCR, affecting the potential of LV to transduce T cells in vitro [17]. In the case of CAR-T cell

production, this is not an issue as cultures are first activated with activator beads.

44. Beads settle very quickly, thus to ensure accurate transfer of the correct number of beads, pipette the required amount immediately after resuspension. Although the manufacturer recommends a ratio of 1:1 bead–T cell ratio, we utilize a slightly lower ratio of 0.8:1. However, the literature has used ratios as high as 3:1 [18], although it should be noted that these cultures utilize a slightly different cytokine cocktail.
45. Often a MOI of 1–10 is sufficient to achieve transduction efficiencies of >60%. This is dependent on the individual virus batch and should be titrated on T cell cultures to determine optimal MOI. We have found that the number of transducing units (as determined by the MOI) added can have a significant impact on T-cell growth. Reporter genes that show no impact on T-cell behavior or growth have demonstrated reduced T-cell proliferation at high MOI's. In our hands, MOI's of 10 and below have consistently shown to have no significant impact.
46. To avoid disturbing the PBMC pellet, tilt the plate slightly forward and remove media carefully from the lower edge. As previously mentioned, cell disruption impacts cell proliferation. Especially in early stages of T-cell growth, a 20–30% reduction in cell proliferation was observed if pellets were disrupted.
47. We have found with this protocol, almost universally the cell pellets are sufficiently large the second day following addition of 100 μL media. In the case that they are not ready by this point, carefully replace half media with fresh cytokine-supplemented media.
48. This step is best completed by bringing a 100 μL micropipettor close to the T cell pellet and gently aspirating. This process maintains the general structure of the pellet and allows you to transfer it over. We have found that minimal disruption is important to the growth of the T cells and we believe at this stage they are interacting with each other and the activator beads. You may use some of the 900 μL media to wash the remainder of the well for any remaining cells.
49. This metric is slightly dependent on the state of the pellet when it was originally transferred to the 24-well plate. That is to say, should the pellet remain completely whole, it will not spread as quickly if the pellet was transferred in several pieces. We have found that on average this takes 2 days. Cells should be given fresh media every 2–3 days, if you deem a well not ready by this point, simply add 1 mL cytokine-supplemented T cell media.
50. We have found as a general rule of thumb that 100,000 cells /cm^2 is a low point for cell density over surface area. Below this number, we have found the proliferation of the T-cells is impacted

negatively. With two wells at ~90% confluence, this is about $2–2.5 \times 10^6$ cells and is sufficient. If you are unsure, it is best to resuspend cells thoroughly and count. However, to promote growth it is still optimal to not disturb cells at this point.

51. Cell densities exceeding 300,000 cells/cm^2 may prove problematic for nutrient and oxygen exchange, thus we recommend maintaining within 100,000–200,000 cells/cm^2. It is important to have the appropriate volume of media within a flask of certain size. This is to maintain optimal oxygenation of the T cells. We have found that media for the T-25, T-75, and T-150 should not exceed 6 mL, 15 mL, and 25 mL, respectively.

Acknowledgements

We would like to kindly thank Dr. Megan Levings for providing the original lentiviral generation protocol and her guidance with which to proceed in lentiviral preparation. We would also like to extend thanks to Dr. Brian Rabinovich for providing both help and plasmids in the generation of retroviruses used for engineering of murine T cells.

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